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PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US89/02172 (22) International Filing Date: 19 May 1989 (19.05.89)</p> <p>(71) Applicant: HEM RESEARCH, INC. [US/US]; 12280 Wilkins Avenue, Rockville, MD 20852 (US).</p> <p>(72) Inventors: GILLESPIE, David, H. ; RD 3, Box 184, Mapleflower Road, Glenmoore, PA 19341 (US). CARTER, William, A. ; 1 Jaine Lane, Birchrunville, PA 19421 (US).</p> <p>(74) Agent: CRAWFORD, Arthur, R.; Nixon & Vanderhye, Fourteenth Floor, 2200 Clarendon Blvd., Arlington, VA 22201 (US).</p>		<p>(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent)*, DK, FI, FR (European patent), GA (OAPI patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: SHORT THERAPEUTIC dsRNA OF DEFINED STRUCTURE</p> <p>(57) Abstract</p> <p>The present invention relates to the induction of proteins and the activation of enzymes in the cells of living organisms, including human beings. According to the invention, long nucleic acid complexes, such as the polyribonucleic acid and polycytidylate complexes, are modified to yield short dsRNA of defined sequence. Said short dsRNAs of defined sequence retain the ability to induce proteins and activate enzymes but are not as toxic as long dsRNA.</p>			

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SHORT THERAPEUTIC dsRNA
OF DEFINED STRUCTURE

BACKGROUND AND SUMMARY

5 The invention generally relates to therapeutic compositions of matter, methods for producing said compositions and methods for administering said compositions to living organisms, including human beings.

10 Certain long, double-stranded RNA (dsRNA), especially poly(I):poly(C) and poly(I):poly (C₁₂,U) (Ampligen®) are anticancer and antiAIDS agents (1). These dsRNAs induce interferon and activate a variety of cellular enzymes (2). These dsRNAs are enzymatically synthesized as high molecular weight 15 nucleic acid polymers (m>300), using ribonucleoside diphosphates as substrate and polynucleotide phosphorylase (PNPase) as enzyme. Ampligen was created because its parent compound, poly(I):poly(C), was toxic (3). In the 1960's, Drs. Carter and Ts'o 20 reasoned that a metabolically unstable, long dsRNA derivative might be cleared quickly from blood and therefore exhibit minimal toxicity, (4). They created Ampligen® as a long dsRNA molecule with RNase-sensitive mismatches and this molecule retained 25 biologic potency while proving to be non-toxic (1). To indicate the size of poly(I):poly(C) or poly(I):poly (C₁₂,U) in general use, the inventors point out that the specifications for Ampligen in 30 clinical trials in 198701988 include a requirement of an S20, w of 10-15, corresponding to a molecular

weight in excess of 1,000,000 and a number of base pairs in excess of 1500.

A new line of thinking has lead the present inventors to a new and different mechanism account for biological activity and lack of toxicity simultaneously characterizing dsRNA, allowing the inventors to create a new class of dsRNA molecule with therapeutic benefit. In contrast to the teachings of the prior art, the inventors have concluded that lack of toxicity of Ampligen derives from the helical interruptions (i.e., shortness of helical stretches) introduced by uracil residues and not from RNase sensitivity. Thus, the essence of this invention is as follows: First, certain short dsRNAs will be nontoxic and biologically active, regardless of whether they contain RNase-sensitive mismatches. Such short dsRNAs having the proper nucleotide sequence will be therapeutic by virtue of their biological activity. It should be emphasized that the prior art teaches away from the present invention by teaching the need for long, biodegradable dsRNA in order to preserve biological activity without toxicity.

Short dsRNA of defined sequence cannot be synthesized by the PNPase method. First, PNPase cannot synthesize a nucleic acid of defined sequence because it is a terminal transferase and not a template-copying enzyme. Second, the helical content of dsRNAs of the homopolymer:homopolymer type constantly changes due to the "slippage" reaction. Slippage means that the two strands of the dsRNA

5 molecule mover relative to each other since there is no complementary nucleotide register to fix the position of two strands relative to each other. The present invention produces means for synthesizing stable short dsRNA of defined sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

10 FIG. 1 is an illustration of two types of short dsRNA of defined sequence. In FIG. 1A is shown a dsRNA with terminal "locks" while in FIG. 1B is shown a dsRNA with a central "hinge". The dsRNA in FIG. 1 also contain "internal registers", which are indicated by A-U base pairs.

15 FIG. 2 is an illustration of a method for preparing a short dsRNA of defined sequence having terminal locks and internal registers.

FIG. 3 is an illustration of a method for preparing short dsRNA of defined sequence having a central or near-central hinge.

20 FIG. 4 is an illustration of a method for preparing short dsRNA of defined sequence having both terminal locks and a central or near-central hinge.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

25 1) Types of dsRNA. The present compositions of matter and methods relative to their use generally depend on the several embodiments thereof on the chemical modifications of protein-inducing and/or

enzyme-activating dsRNA complex to render said complex less toxic to a living animal cell. The chemically modified complexes disclosed herein retain the biological activity of unmodified complexes while being less toxic by mechanisms which are presently mysterious. The dsRNA complexes which are of concern in the present invention may be modified by shortening said dsRNA while at the same time fixing the two strands in space relative to one another.

5 Figure 1 depicts two types of dsRNA envisioned in this application. Type A dsRNA is termed "locked dsRNA". It contains complementary regions at each end ("locks") to fix the dsRNA register. Variants of this model may contain subterminal, rather than or in addition to, terminal locks or may contain only one lock. Locks may be as short as a single nucleotide. In addition, "internal registers" like the A-U base pairs of Figures 1 and 2 may be added for increased stability. Type B dsRNA is termed "hinged" dsRNA.

10 15 20 25 30 Hinged dsRNA contains an internal self-complementary stretch which folds in a restricted way to align the remaining dsRNA nucleotides (see also Figures 3 and 4 for example of hinged dsRNA). For the purposes of this application "locks", "hinges" and "internal registers" are complementary nucleotide pairs different from homopolymer stretches in the dsRNA. Said locks, hinges and internal registers are referred to as heteropolymer regions in this application. At one extreme is a homopolymer with a single heteropolymer nucleotide pair to fix the dsRNA register while at the other extreme is a totally heteropolymeric short dsRNA. Both locked and hinged dsRNAs may contain single stranded regions terminally

or internally (Figure 2).

2) Synthesis of Short dsRNAs of Defined

Sequence. Locked RNA molecules of defined length and defined sequence can be synthesized from plasmid DNA vectors having promoters of defined sequence placed near the sequence of interest. The vectors, enzymes and substrates are available from a variety of commercial sources. For example the locked dsRNA depicted as structure [7] of Figure 2 can be made as follows. The two deoxyoligonucleotides depicted at the top of Figure 2 (structure [1]) can be synthesized by an oligonucleotide synthesizer. Annealing them as shown (structure [2]) leaves single-stranded ends which can be cloned into the commercially available vector, pGEM 4, after cleavage of the vector with EcoRI and Hind III, yielding structure [3]. Transcription of this plasmid as described in Figure 2 yields separately two single stranded RNAs (structures [4] and [5]) which can be annealed (e.g., at 65° in 1M NaPO4, pH7) to produce the dsRNA shown in structure [6] in Figure 2. This locked dsRNA can be used as is or can be trimmed with RNase to produce the dsRNA shown in structure [7], Figure 2.

25 Hinged RNA molecules of defined length and defined sequence can be synthesized from plasmid DNA vectors having promoters of defined sequence placed near the sequence of interest. For example, the hinged dsRNA depicted as structure [8] of Figure 3 can be made as follows: The two deoxyoligonucleotides depicted at the top of Figure 3

(structures[1]) can be synthesized by an oligonucleotide synthesizer, annealed to produce structure [2] and cloned into pGEM4 previously cut with Eco R1 and Sma 1 yielding structure [3].

5 Transcription of this plasmid as described in Figure 3 yields an RNA (structure [4] which can be self-annealed to produce the dsRNA shown as structure [5]. This hinged dsRNA can be used as is or can be trimmed with RNase to produce the dsRNA shown as 10 structure ([6]), Figure 3. dsRNA molecules of defined length and sequence with both locks and hinges can be synthesized by a slight modification of this above procedure, using Hind II instead of Sma 1 and using slightly different deoxyoligonucleotides, 15 as depicted in Figure 4.

It will be obvious to those with ordinary skill in the art that other vectors and other restriction endonuclease sites can be used with similar results. It will also be obvious that other degrees of 20 repetition than 3 of $[(I_{10})A]/[(C_{10})U]$ can be employed and that other polypurine/polypyrimidine tracts can be used, such as $(I_n)/(C_n)$, $(I)_n/[(C);U]_n$, $(A)_m/(U)_m$, etc., so long as helical regions are kept short enough to avoid toxicity and long enough to 25 retain biological activity.

It will also be obvious to those with ordinary skill in the art that oligonucleotides consisting of RNA polymerase promoters flanking inserts specifying the present invention can be synthesized, annealed 30 and transcribed directly, without cloning into a vector. It will also be obvious to those with

ordinary skill in the art that short dsRNA can be chemically synthesized. The essence of this invention lies in the structure and properties of the dsRNAs themselves; this example is given to enable one with ordinary skill in the art to prepare short therapeutic dsRNAs of defined sequence.

3) Determining Biological Activity of Short dsRNA of Defined Sequence. The biological activity of dsRNA can be assessed in several experimental systems which are routine in the art. The antiviral properties of dsRNA can be measured by challenging dsRNA-treated cells with vesicular stomatitis virus (VSV) and measuring reduction in virus yield as described by (5). Similar procedures have been reported which measure the inhibition of VSV and other viruses. The antitumor properties of dsRNA can be evaluated by exposing tumor cells in tissue culture to dsRNA and measuring reduction in growth rate as described by (6). The antitumor properties of dsRNA can also be measured by injecting dsRNA into nude mice bearing tumors and measuring tumor growth rate (7). The ability of dsRNA to enhance natural killer cell or macrophage killing activity can be determined as detailed (8). All of these procedures are routine in the art and are cited by way of enabling one with ordinary skill in the art to measure the biological activity of dsRNA synthesized as described in the preceding section. The citing of these procedures should not be construed as limiting; other procedures for measuring the biological activity of dsRNA exist and are also well known in the art.

4) Determining the Absence of Toxicity of dsRNA of Defined Sequence. The toxicity or lack of it of dsRNA can be determined by procedures which have long been routine for testing a variety of potential 5 therapeutics and which have long been routine for testing a variety of potential therapeutics and which have been applied to dsRNA as well. Suitable test animals such as mice, rats, rabbits, dogs, monkeys, etc. or humans can be injected periodically with 10 various quantities of dsRNA and after a suitable interval, such animals can be examined for evidence of fever, loss of weight, loss of liver function, thrombocytopenia, leukopenia, bone marrow suppression, etc. The examiner is directed 15 particularly to Citations (9) for examples where such studies have been done regarding dsRNA.

WHAT IS CLAIMED IS:

1 1. Short dsRNA of defined structure, said dsRNA
2 having biological activity and lacking significant
3 toxicity.

1 2. The dsRNA of Claim 1 stabilized by "lock",
2 "hinge" and/or "internal register" regions of
3 complementary heteropolymer.

1 3. The dsRNA of Claim 2 having the general
2 structure:

3 . 5'lock-(I)_n-lock 3'
4 . 3'lock-(C)_m-lock 5'

5 where said "m" and said "n" are less than 100
6 and more than 5 and where said locks in one strand
7 are complementary to locks in the opposite strand and
8 where said "I" and said "C" are inosine monophosphate
9 and cytidine monophosphate, respectively.

1 4. The dsRNA of Claim 2 having the general
2 structure 5'(I)_n-hinge-(C)_m3', where said "m" and
3 said "n" are less than 100 and more than 5 and where
4 said hinge is a heteropolymeric region exhibiting
5 self-complementarity and where said "I" and said "C"
6 are inosine monophosphate and cytidine monophosphate
7 respectively.

1 5. The dsRNAs of any one of Claims 1-5 having the
2 general structure

3 5' lock-[(I)_nA]_j-lock 3'
4 3' lock-[(C)_mU]_k-lock 3'

5 where said "m" and said "n" are less than 25 and more
6 than 5, where said "j" and said "k" are less than 10
7 and more than 0, where said "I" and said "C" are
8 inosine monophosphate and cytidine monophosphate,
9 respectively, where said "A" is a nucleotide which is
10 not I and where "U" is a nucleotide which base pairs
11 with said A.

1 7. The dsRNA of Claim 1 with substitutions in one
2 strand, said substitutions being not complementary to
3 nucleotides in the opposite strand.

1 8. The dsRNA of Claim 7, said dsRNAs having
2 single-stranded tails.

1 9. A method of therapeutically activating
2 dsRNA-dependent enzymes or inducing interferon in a
3 human in need of such therapy, which method comprises
4 administering to the human a therapeutically
5 effective amount of the short dsRNA of defined
6 structure according to claim 1.

1 10. A method of therapeutically activating
2 dsRNA-dependent enzymes or inducing interferon in a
3 human in need of such therapy, which method comprises
4 administering to the human a therapeutically
5 effective amount of the short dsRNA of defined
6 structure according to claim 2.

1 11. A method of therapeutically activating

2 dsRNA dependent enzymes or inducing interferon in a
3 human in need of such therapy, which method comprises
4 administering to the human a therapeutically
5 effective amount of the short dsRNA of defined
6 structure according to claim 3.

1 12. A method of therapeutically activating
2 dsRNA dependent enzymes or inducing interferon in a
3 human in need of such therapy, which method comprises
4 administering to the human a therapeutically
5 effective amount of the short dsRNA of defined
6 structure according to claim 4.

1 13. A method of therapeutically activating
2 dsRNA dependent enzymes or inducing interferon in a
3 human in need of such therapy, which method comprises
4 administering to the human a therapeutically
5 effective amount of the short dsRNA of defined
6 structure according to claim 5.

1 14. A method of therapeutically activating
2 dsRNA dependent enzymes or inducing interferon in a
3 human in need of such therapy, which method comprises
4 administering to the human a therapeutically
5 effective amount of the short dsRNA of defined
6 structure according to claim 6.

1 15. A method of therapeutically activating
2 dsRNA dependent enzymes or inducing interferon in a
3 human in need of such therapy, which method comprises
4 administering to the human a therapeutically
5 effective amount of the short dsRNA of defined
6 structure according to claim 7.

1 16. A method of therapeutically activating
2 dsRNA dependent enzymes or inducing interferon in a
3 human in need of such therapy, which method comprises
4 administering to the human a therapeutically
5 effective amount of the short dsRNA of defined
6 structure according to claim 8.

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FIG. 1A

5'lock-III.....III.....III.....III.....lock 3
 3'lock-CCCCCCCCCCCCCCCCCCCCCCCCCUU.....lock 5

or 5'lock-[$(I)_{10}^A$]₃-lock 3'
 3'lock-[$(C)_{10}^U$]₃-lock 5'

or, generally 5'lock-(N')_n-lock 3'
 3'lock-(N')_m-lock 5'

FIG. 1B

hinge-III.....III.....III.....
 hinge-CCCCCCCCCCCCCCCCCCCC5'

or hinge-(I)₁₀^U(I)₁₀³'
 hinge-(C)₁₀A(C)₁₀⁵'

or, generally hinge-(N')_n³'
 hinge-(N')_m⁵'

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FIG. 2

make deoxyoligonucleotides 5'AGCTTACCC [(G)₁₀A]₃G and
ATGGG [(C)₁₀T]₃CTTAA5'

anneal oligonucleotides
5'AGCTTACCC [(G)₁₀A]₃G
ATGGG [(C)₁₀T]₃CTTAA5'

clone annealed oligos into pGEM4 cut with
EcoRI and Hind III

SP6 GAATACAAGCTTACCC [(G)₁₀A]₃ GAATTCCCGGTCTCCC T7
PROMOTORCTTATGTTCGAATGGG [(C)₁₀U]₃ CTTAAGGGCCAGAGGG PROMOTOR

cut with Hind III (,) and transcribe with
T7 polymerase plus NTP*

UC1AA [(C)₁₀U]₃CUUAAIIICCAIAIII5' (+) RNA

cut with EcoRI (*, *) and transcribe with
SP6 polymerase plus NTP*

IAAUACAAICUU [(I)₁₀A]₃IAAUU3' (-) RNA

anneal (+) and (-) RNA

UC1AAUIII [(C)₁₀U]₃CUUAAIIICCAIAIII 5'
IAAUACAAICUUACCC [(I)₁₀A]₃IAAUU

RNase T2

UC1AAUIII [(C)₁₀U]₃CUUAA
A1CUUACCC [(I)₁₀A]₃IAAUU

NTP* = ATP, UTP, CTP plus ITP

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FIG. 3

make deoxyoligonucleotides $GGG(G)_{12}AATGATT(C)_{15}G$ and
 $CCC(C)_{12}TTACTAA(G)_{15}CTTAAG$

anneal oligonucleotides

$GGG(G)_{12}AATGATT(C)_{15}G$
 $CCC(C)_{12}TTACTAA(G)_{15}CTTAAG$

clone annealed oligos into pGEM4 cut with EcoRI and SmaI

SP6 POLY $CCCGGG(G)_{12}AATGATT(C)_{15}GAATTCCGGTCTCCC$ T7
PRO LINKER $GGGCC(C)_{12}TTACTAA(G)_{15}CTTAAGGCCAGAGGG$ PRO

cut with SmaI (.) and transcribe with T7 polymerase plus NTP*

$(C)_{15}UUACUAA(I)_{15}CUUAAIICCAIAIII5'$

self anneal

$UAA(I)_{15}CUUAAIICCAIAIII5'$
 $AUU(C)_{15}$

RNase A

$UAAIIIIIIIIIIII5'$
 $AUUCCCCCCCCCCCCC$

NTP* = ATP, UTP, CTP plus ITP

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FIG. 4

make deoxyoligonucleotides AGCTT(G)₁₂AATGATT(C)₁₂AAGCTG
and A(C)₁₂TTACTAA(G)₁₂TTCG

anneal oligonucleotides

AGCTT(G)₁₂AATGATT(C)₁₂AAGCTG
A(C)₁₂TTACTAA(G)₁₂TTCG

clone annealed oligos into pGEM4 cut with EcoRI and Hind III
SP6 GAATACAAAGCTT(G)₁₂AATGATT(C)₁₂AAGCTGAAATTCCGGTCTCCCC T7
PROMOTORCTTATGTCGAA(C)₁₂TTACTAA(G)₁₂TTCGACTAAAGGCCAGAGGGPROMOTOR

cut with Hind III (,) and transcribe with T7 polymerase plus NTP*
UCIAA(C)₁₂UUACUAA(I)₁₂UUUCIACUUAAUCCAAIIII5'

self anneal RNA

UAA(I)₁₂UUUCIACUUAAUCCAAIIII5'
AUU(C)₁₂AAICU

RNase A.

UAAIIIIIIIIUUUCIA5'
AUUCCCCCCCCCCAAAICU

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02172

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶	
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4th Ed.): A61K 31/70; C07H 19/067; A61K 45/02 U.S. Cl.: 536/27; 536/28; 536/29; 424/85.4-85.7	

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
U.S. Cl.	536/27; 536/28; 536/29; 424/85.4-85.7

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	N "Augmenting Agents in Cancer Therapy", Issued 1981, Raven Press, (New York, New York, USA), W. A. Carter et al. "Poly IC with Mismatched Bases, Prospects for Cancer Therapy", see pp. 177-183.	1-16
Y	N Nature, vol. 297, Issued 03 June 1982, (London, England), S. L. Lin et al., "Sensitivity and resistance of human tumour cells to interferon and rI _n · rC _n ", see pp. 417-419.	1-16
Y	N Molecular Pharmacology, vol. 15, Issued 1979 (New York, New York, USA), J. A. O'Malley et al., "Polyinosinic Acid-Polycytidylic Acid and Its Mismatched Analogues: Differential Effects on Human Cell Function", see pp. 165-173.	1-16
Y	N Cancer Research, vol. 46, Issued April 1986(Bethesda, Maryland, USA), M. S. Chapekar et al., "Potentiation of the Cytocidal Effect of Human Immune Interferon by Different Synthetic Double-Stranded RNAs in the Refractory Human Colon Carcinoma Cell Line BE", see pp. 1698-1702.	1-16

- Special categories of cited documents: ¹⁴
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "I" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "V" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 16 August 1989	Date of Mailing of this International Search Report 07 SEP 1989
International Searching Authority ISA/US	Signature of Authorized Officer L. Eric Crane <i>L. Eric Crane</i>

Form PCTISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,400,375, DOUHART ET AL., Published 23 August 1983, see columns 1-10.	1-16
Y	US, A, 4,388,506, FIELD ET AL., Published 14 June 1983, see columns 1-6.	1-16
Y	GB, B, 1,411,748, VERE-HODGE, Published 29 October 1975, see pages 1-12.	1-16
Y	SU, B, 425,940, MACROMOLECULAR INSTITUTE ET AL., Published 30 April 1974, see Derwent English language abstract 70156W/42.	1-16
Y	US, A, 4,262,090, COLBY ET AL., Published 14 April 1981, see columns 1-14.	1-16

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	N Chemical Abstracts, Vol. 107, Issued 1987(Columbus, Ohio, USA), D. C. Montefiori et al., "Antiviral activity of mismatched double-stranded RNA against human immunodeficiency virus in vitro.", see abstract number 51430a on p. 21.	1-16
Y	N Journal of Biological Chemistry, vol. 254, Issued 25 October 1979 (Easton, Pennsylvania, USA), M. A. Minks et al, "Structural requirements of double-stranded RNA for the activation of 2',5'-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells.", see pp. 10180-10183.	1-16

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter² not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out³, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(e).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING⁴

This International Searching Authority found multiple inventions in this International application as follows:

1. As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.
2. As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	N Acta Virology, Vol 14, Issued 1970, I. Rosztoczy et al., "Enhancement of Interferon Synthesis by Polyinosinic-Polycytidylc Acid in L Cells Pretreated with Interferon.", see pp. 398-400.	1-16
Y	N Chemical Abstracts, Vol. 108, Issued 1988(Columbus, Ohio, USA), H. R. Hubbell et al. "Augmented antitumor effect of combined human natural interferon-alpha and mismatched double-stranded RNA treatment against human malignant melanoma xenograft.", see abstract number 4453w on p 434.	1-16
Y	N Proceedings of the National Academy of Science USA, Issued 1967 (Washington, D. C. USA), A. K. Field et al., "Inducers of Interferon and Host Resistance, II. Multistranded Synthetic Polynucleotide Complexes.", see pp. 1004-1010.	1-16
Y	US. A. 4,313,938, ARIMURA ET AL., Published 02 February 1982, see columns 1-20.	1-16
Y	US. A. 4,349,538, LEVY, Published 14 September 1982, see columns 1-8.	1-16
Y	US. A. 4,024,241, LEVY, Published 17 May 1977, see columns 1-6.	1-16
Y	US. A. 4,018,916, HODGE, Published 19 April 1977, see columns 1-10.	1-16
Y	US. A. 3,819,482, VIDAVER ET AL., Published 25 June 1974, see columns 1-12.	1-16
Y	US. A. 4,767,701, HOLMBERG ET AL., Published 30 August 1988, see columns 1-6.	1-16
Y	US. A. 4,130,641, T'SO ET AL., Published 19 December 1978, see columns 1-22.	1-16
Y	US. A. 4,124,702, LAMPSON ET AL., Published 07 November 1978, see columns 1-30.	1-16
Y	US. A. 4,024,222, T'SO ET AL., Published 17 May 1977, see columns 1-20.	1-16
Y	US. A. 3,679,654, MAES, Published 25 July 1972, see columns 1-6.	1-16
Y	US. A. 3,444,043, SPIEGELMAN, Published 13 May 1969, see columns 1-12.	1-16
Y	US. A. 3,660,564, YONEDA ET AL., Published 02 May 1972, see columns 1-12.	1-16